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Oiginal

Seasonal Fluctuation on the Proteins in the Bark of *Sophora japonica* L. Analyzed by Enzyme Immuno-Assay.

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Abstract——Electrophoretical analysis showed that four proteins were existed in winter and disappeared in summer in the bark of Sophora japonica. The two major proteins of them were purified and the antibodies were raised against them. The seasonal changes of them were measured by enzyme immuno-assay, and the profiles of them were compared. The two profiles were quite different, especially in autumn. One increased linearly from summer to winter, whereas the other increased with fluctuation during the same period. These results suggested that these proteins may have the distinct roles in the bark.

Keywords: Bark (phloem) proteins, enzyme immuno-assay, seasonal change

1. Introduction

The seasonal fluctuating proteins may be related to the important factor of tree physiology. The overground tissues of trees, especially the stems, are alive through all the seasons, indicating that they highly adapt themselves to surroundings with keeping their lives. At least, tree stems have a potential to cope with the seasonal changes of the climate. It is assumed that the adaptation to the climate should be accompanied with functional changes of the tissues. The changes should be reflected on the changes in amount or species of proteins.

In the earlier studies, the changes in the amount of total protein in the bark was shown as being more in winter than in summer[1]. Recently, however, few proteins were identified as the seasonally fluctuating proteins. One is a protein termed lectin, and the others are not characterized except for their molecular sizes. The former was shown to ocurr in the bark of *Sophora japonica*[2], *Robinia pseudoacacia* and *Sambucus nigra*[3], the latter was found in the bark of *Populus deltoides*, *Salix smithiana* and *Acer saccharum*[4].

Lectin is a dominant protein in the bark of *Sophora japonica* and its amount changes

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seasonally: it increases in autumn, the high level in amount is maintained during winter, and it decreases in spring. However, there is a possibility that the other seasonal fluctuating proteins coexist with lectin in the same bark. It will contribute further understanding of tree physiology to clarify how many proteins are involved in the seasonal changes, and how these seasonal changes ocurr through a year.

The present study was designed to identify the other seasonal fluctuating proteins within the bark of *Sophora japonica,* and to determine their annual changes quantitatively. The proteins which dissappear in summer are focused. In order to investigate such proteins, the electrophretical migration patterns of winter and summer were compared on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The proteins found on the PAGEs were purified, and the antibodies were raised against them. The seasonal changes of their levels were measured by enzyme-linked immunosorbent assay (ELISA) on monthly basis.

2. Material and methods

2.1 Plant material

An individual *Sophora japonica* tree on the campus of Wood Reseach Institute, Kyoto university was used. Bark specimens were harvested monthly from the main stem (about 15 cm in diameter) and immediately put into liquid nitrogen. They were stored at −80°C until use.

2.2 Sodium dodecylsulfate polyacrylamide gel electrophoresis

The bark samples harvested in June and in December were used. They were homogenized with 125 mM Tris buffer containing 8 M urea and 5% sodium dodecylsulfate (SDS), sonicated with a Sonifier Cell Disrupter 200 (Branson, Danbury, Conn., USA) and centrifuged at 10,000·*g* for 15 min. The protein contents of the supernatants were determined using the bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, Ill., USA). Then, β-mercaptoethanol was added to 5% concentration and the aliquots were boiled for 5 min, followed by centrifugation at 10,000·*g* for 15 min. The supernatants were charged as equal protein amounts on 12% polyacrylamide gel (9·6·0.1 cm^3) and subjected to electrophoresis using the discontinuous buffer system of Laemmli[5]. The proteins in the gel were stained with Coomassie brilliant blue R250 (CBB; Nacalai Tesque), and then the density of the each lane was measured by an image analyzer on the densitometric condition (Ibas 20; Zeiss, Germany).

2.3 Two-dimensional polyacrylamide gel electrophoresis

Each specimen was cut into about 1 mm^3 cubes and put into an Eppendorf tube with lysis buffer containing 8 M urea, 2% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo., USA), 5% β-mercaptoethanol, 0.5% SDS. After squashed with glass-rod, they were

sonicated by using a Sonifier Cell Disruptor 200 (Branson, Danbury, Conn, USA). The resulting suspension was centrifuged at 10,000·*g* for 15 min. The supernatant was recovered as the extracts. The extracts were analyzed by 2D-PAGE according to the method of O'Ferell[6]. The isoelectric focusing gel contained 2% ampholine (one part of pH 3.5–10 and one part of pH 5–7) (LKB, Sweden). For second dimension, 12% polyacrylamide gel was used.

2.4 Antiserum

The protein spots in the 2D-PAGE gels stained with CBB were cut off and electrically eluted from the gel by using an electro-eluter (Nippon Eido, Tokyo, Japan). After dialyzed against distilled water, the eluted proteins were lyophilized. They were dissolved into PBS, and emulsified with Freund's complete adjuvant. The resulting emulsions were injected under the skin of rabbits. After following four times injection using incomplete adjuvant instead of the complete adjuvant week by week, the whole blood of each rabbit was collected a week after the last injection. After leaving at room temperature at least 30 min, they were centrifuged at 500·*g* for 20 min, and antiserum against each protein was obtained.

2.5 Enzyme linked immunosorbent assay

To each sample, harvested monthly, 0.1 M carbonate buffer (pH 9.7) was added in 1 ml/mg fresh weight. They were sonicated and centrifuged. Each supernatant was diluted in ten stages linearly from 0- to 10,000-folds and 50 ml of each was applied to 96-well EIA plates (Coster, Boston, Mass., USA) and left at 4°C overnight. The wells of the plates were then washed with 0.05% Tween 20 in 0.1 M phosphate buffered saline (TPBS) three times, and blocked with 1% BSA in PBS for 30 min. After blocking, antiserum diluted in 100-fold was applied, and the preparations incubated for 1 h. After this they were washed with

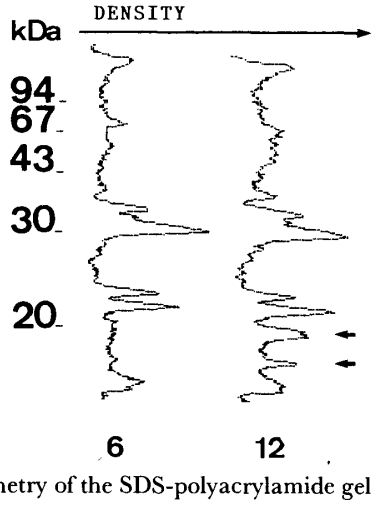

Fig. 1. Densitometry of the SDS-polyacrylamide gel electrophoresis of the bark proteins of *Sophora japonica*.
Lane 6, harvested in June; **Lane 12**, harvested in December.
Arrows indicate the peak of 20 and 18 kDa observed only in December.

TPBS three times and peroxidase-conjugated anti-rabbit antibody (Bio-Rad, Richmond, Va., USA), diluted 3000-fold, was applied, followed by another incubation for 1 h. The wells were then washed again three times with TPBS; color was developed with *o*-phenylenediamine (Wako Pure Chemical Industries, Osaka) and absorbance was measured at 492 nm.

3. Results and Discussion

Figure 1 shows the major changes occurred in the bark proteins of *Sophora japonica* between summer (June) and winter (December), analyzed by densitometry of the migration of SDS-PAGE. The most obvious change was that the peaks of 20 kDa and 18 kDa in apparent molecular sizes were present only in December. In order to search for the other

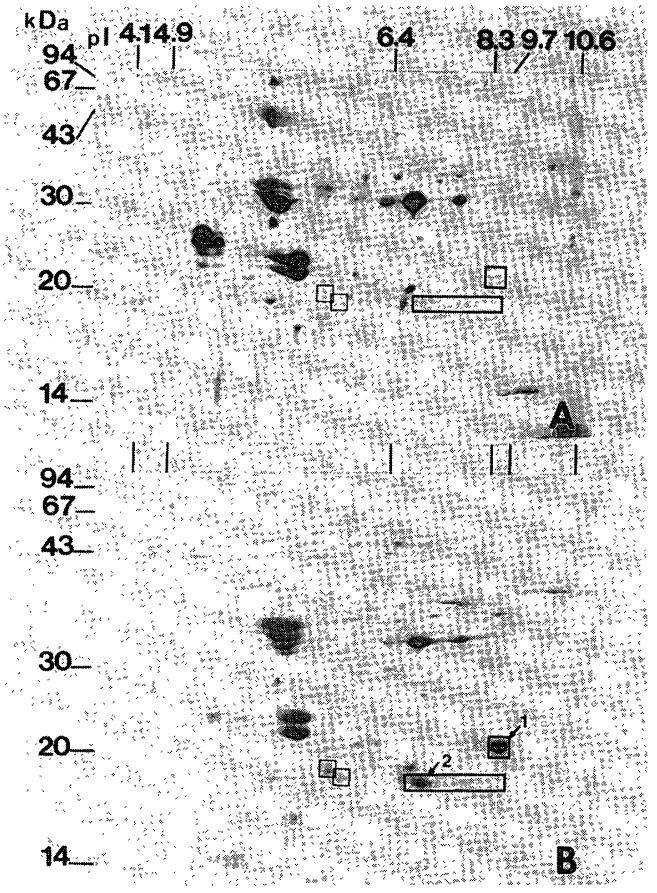

Fig. 2A, B. Two-dimensional polyacrylamide gel electrophoresis of the bark proteins of *Sophora japonica*.
A: harvested in June; **B**: harvested in December. The protein spots marked with square and rectangle are observed only in December (B). The same positions on the gel of June are also marked (A). The proteins indicated by arrows, 1, WP20 and 2, WP18 were purified. Antibodies against them were raised.

changes in relation to season, the bark proteins in June and in December were analyzed by 2D-PAGE (Figure 2). The 18 kDa band, found on SDS-PAGE, was not a single protein, but observed as a group of 3 or more protein spots spreading in pI=7.0–8.3. Other than the group of 18 kDa, three protein spots were found as the obvious changes appearing in December and disappearing in June: 20 kDa, pI 9.1, 18 kDa, pI 6.3, and 19 kDa, pI 6.2. Among these proteins, two proteins were purified and antiserum against them were obtained; one was the protein of 20 kDa (arrow 1 in Fig. 2B), and the other was the group of 18 kDa (arrow 2 in Fig. 2B), because they gave larger spots on the gel. The former was named WP20 (winter protein of 20 kDa), the latter WP18. Each antiserum was checked that they recognized only WP20 and WP18, respectively, on the western blotting of 2-D PAGE gel.

By using each antiserum, the annual changes of their amounts were measured on monthly basis (Figs. 3, 4). The contents per fresh weight of each month was shown as relative value of the most abundant month, March. Both of them were abundant in winter and less in summer, but the changing profiles were somewhat different from each other. WP20 decreased linearly from March until July when it disappeared completely, and increased linearly until the next March (Fig. 2). WP18 decreased from March to June as well as WP20, however, it did not completely disappear even in summer, and was retained

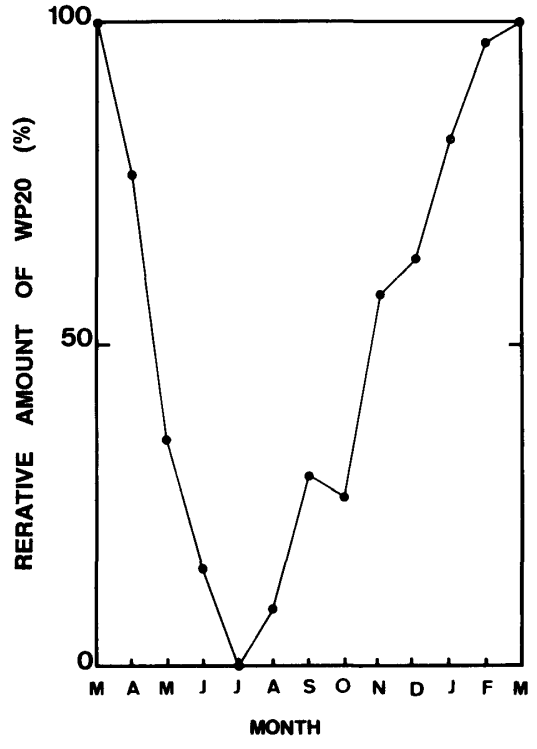

Fig. 3. Seasonal changing profile of WP20 measured by enzyme linked immunosorbent assay.

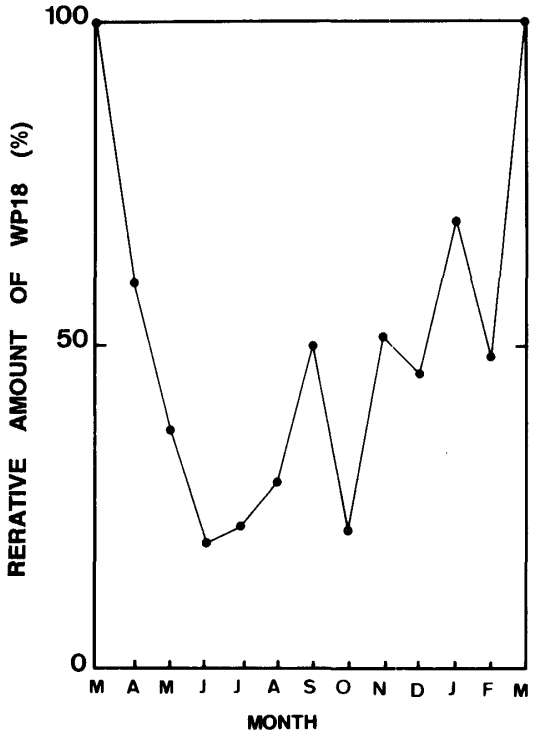

Fig. 4. Seasonal changing profile of WP18 measured by enzyme linked immunosorbent assay.

about 20% of the maximum level. Then in autumn, the WP18-level was kept about 50% with a little fluctuation, and increased with a large fluctuation from the end of autumn. By the observation on 2-D PAGE, WP 18 was apparently absent in summer, but the results on ELISA showed that it was present even in summer. Perhaps the amount in summer was too little to be detected by the staining with Coomassie brilliant blue.

Although the profiles of WP20 and WP18 were dissimilar especially in their increasing period, both of them were abundant in winter and less in summer. Furthermore, the difference of the level between winter and summer of them were greater than that of the lectin. The amount changes of WP20 and WP18 were distinguishable by PAGE level, whereas the change in the lectin was not found until the measurement by ELISA[2]. WP20 was undetectable in July and WP18 was about 20% of the most abundant month, while the lectin retained about 50%. Total proteins in bark have been known to be more abundant in winter than in summer[1]. Recently, it was reported that several protein species show such seasonal changes[2–4]. Two roles are thought to be played by such proteins. One is storage of nitrogen during the dormant period[3,4,7,9], and the other is the role for cold resistance in winter[8]. WP20 and WP18 would play either of the two. This is the first report for the measurement of the changes in the certain bark proteins other than lectin, and showing their profiles in relation to season. According to the results in this paper, it was demonstrated that the annual profiles are varied with the protein species, even if they are present in winter and dissapear in summer.

References

1) P.J. Kramer and T.T. Kozlowski : In : Physiology of woody plants, pp. 304–314. Academic Press Inc., New York/San Francisco/London (1979).
2) K. Baba, M. Ogawa, A. Nagano, H. Kuroda, and K. Sumiya : *Planta,* **183**, 462–470 (1991).
3) M. Nsimba-Lubaki and W.J. Peumans : *Plant physiol.,* **80**, 747–751 (1986).
4) S. Wetzel, C. Demmers and J.S. Greenwood : *Planta,* **178**, 275–281 (1989).
5) U.K. Laemmli : *Nature,* **227**, 680–685 (1970).
6) P.H. O'Ferell : *J. Biol. Chem.,* **250**, 4007–4021 (1975).
7) B.T. O'Kennedy and J.S. Titus : *Physiol. Plant.,* **45**, 419–424 (1979).
8) S. Kang and J.S. : *J. Hort. Sci.,* **62**, 281–285 (1987).
9) J.J. Sauter and B. Cleve : *Planta,* **183**, 92–100 (1990).